INFLUENCE OF BCO2 ON GENE EXPRESSION IN MICE HEPATOCYTES

By

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IN MICE HEPATOCYTES

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Introduction

β, β-carotene-9', 10'-dioxygenase 2 (BCO2 or BCDO2) is commonly identified as one of two carotenoid cleavage enzymes that have been discovered in humans [1]. Carotenoids are isoprenoid lipids that serve important physiological functions such as precursors to vitamin A, antioxidants, and light filters. They are thought to be useful biomarkers of disease state and their intake has often been associated with decreased risk of chronic disease in observational studies, though more thorough testing needs to be performed in order to confirm the exact mechanisms that lead to this decrease [2]. Carotenoids are usually made up of eight five-carbon isoprene units for a total of 40 carbon atoms [3]. The central backbone of these structures is an all-*trans* isomer, with a "long conjugated carbon skeleton." This skeleton supports the "main biological property of carotenoids", the scavenging of free radicals [3].

There are two major groups of carotenoids, the carotenes and the xanthophylls. The carotenes are exclusively hydrocarbons, while the xanthophylls are also partially composed of oxygen and are the result of the oxidation of carotenes [4]. Two examples of xanthophylls are lutein and zeaxanthin. These xanthophylls are both cleaved by BCO2 [5]. Lutein and zeaxanthin are found in much higher concentrations in tissues than they are in serum, suggesting that they undergo an active transport process [2]. They are believed by some to be "conditionally essential nutrients," although there are currently no official dietary guidelines for carotenoid intake [4]. Their role in human health is still being investigated, but even in high doses, carotenoids have typically not been found to have harmful, toxic effects on those who consume them as supplements [4]. Because carotenoids serve as precursors for vitamin A biosynthesis, problems with carotenoid

cleavage can lead to a deficiency in vitamin A, which can result in various health complications such as impaired iron metabolism, growth retardation, depressed immune responses, and increased susceptibility to infectious diseases [6]. Carotenoids are potent lipid-based antioxidants as well as anti-inflammatories for the cytokine interleukin-6 and other inflammatory markers [7]. Because BCO2 is involved in carotenoid cleavage, it also is responsible for these defensive properties.

BCO2 is present in large quantities in liver hepatocytes. The enzyme is located exclusively in the mitochondrial inner membrane of these liver cells [1]. It is responsible for the asymmetric cleavage of carotenoids, but unlike β-carotene 15,15'-monooxygenase (BCO1), BCO2 has been found in additional locations that do not typically experience vitamin A deficiency such as in skeletal and cardiac muscle, the endocrine pancreas, and prostate and endometrial connective tissues [8]. Because BCO2 has been found in these areas, it is reasonable to believe that the enzyme may be involved in activities besides the synthesis of vitamin A. BCO2 and BCO1 are part of a "superfamily of non-heme iron-containing oxygenases" of which many, including BCO2, have various functions that have yet to be identified [8]. One study found that BCO2 is considered a "gatekeeper of mitochondrial function" and its absence puts liver cells at risk for increased oxidative stress [9]. Another study found that BCO2 deficient mice exhibited higher levels of carotenoid-induced stress, identifying the enzyme as a key defender against "mitochondrial dysfunction that can result in oxidative stress and disease" [10]. Mitochondria are "critically involved in diabetes" and are targets for hyperglycemia [11]. These diseases are related to inflammation and oxidative stress, suggesting that mitochondrial health is important in defending against conditions like these. Since BCO2 has been found to aide in

mitochondrial health, lack of BCO2 could put individuals at risk for developing a health-threatening condition.

In this study, the effects of BCO2 on mice that have consumed both low-fat diets and high-fat diets will be examined. The study will look at roles of BCO2 in liver cells and will examine the effects of BCO2 knockout on enzymes that have not been previously studied in relation to BCO2.

Methodology

Animal Samples

Initially, eight mice liver samples of approximately 200 mg each were weighed out. These samples were taken from 6-week-old mice that were cared for at the Oklahoma State University Animal Resources Facility. Before being sacrificed, the mice had fasted for five hours. The mice in the study were either wild-type 129S6 or BCO2 knockout. Two liver samples were used from each of the four different types of mice in the study including, high-fat wild type (HF/WT), high-fat knockout (HF/KO), low-fat wild type (LT/WT), and low-fat knockout (LF/KO). In the low-fat (control) diet, 10% of the total kcal consumed by the mice were from fat and in the high-fat diet, 45% of the total kcal consumed were from fat. The animals had been fed the high-fat or low-fat diets for 4 weeks when this experiment was conducted.

Isolation of Total RNA

The samples were homogenized using 4 mL of RNA STAT-60 per sample. After allowing the homogenate to dissociate, total RNA extraction was performed by adding 800

μL of $CHCl_3$ to the samples. The samples were shaken by hand before being incubated at room temperature for 3 minutes. Finally, the homogenates were spun in a floor centrifuge for 15 minutes at 3,500 rpm and 4° C. After this step, two layers were formed in each of the samples, an aqueous layer and an organic layer. To begin RNA precipitation, the clear aqueous upper layers of the samples were pipetted into nuclease-free tubes. Next, 2 mL of cold isopropanol were added to the samples. The samples were then were briefly mixed using a vortex machine and set on ice for about 10 minutes. For the last step in RNA precipitation, the samples were spun in a tabletop centrifuge for 10 minutes at 10,500 rpm and 4° C. After this step, a white pellet was formed in each tube. Next, the RNA wash was performed. The supernatants were disposed of and 1 mL of 75% EtOH was added. The samples were then mixed using a vortex machine for a short period of time before being spun in a tabletop centrifuge again for 5 minutes at 7,500 x g and 4° C. Afterwards, the EtOH was poured off, and the tubes were inverted so they could air dry for about 10 minutes. 1 mL of nuclease-free water was then added to the tubes before they were incubated on ice for 30 minutes.

Spectrophotometry

After resuspension of the pellets, concentrations were determined using spectrophotometry. 2.5 μL of milli Q water were used to clean and initialize the spectrophotometer. 2.5 μL DEPC water were then used to blank the machine. After ensuring that the RNA-40 setting was selected on the spectrophotometer, 2.5 μL of sample were placed on the machine and measured. Initially, the samples had too much organic contamination and needed to be cleaned up before proceeding. To do this, 150 μL of the

samples were used, along with 1 mL of 100% EtOH, 1.5 µL of 5M NaCl, 1 mL of 75% EtOH, and 75 µL of DEPC. After further purification, spectrophotometry was once again utilized to measure the new concentration of our samples. After the concentration data was collected and printed, milli Q water was used to clean the spectrophotometer.

Gel Electrophoresis

The concentrations found were used to prepare the different samples for agarose gel electrophoresis. An agarose gel was made using 0.45 g of agarose powder and 4.5 mL of TAE. This mixture was microwaved until the agarose was dissolved and was then allowed to stand for about 10 minutes. Once the mixture was cool enough, 4.5 µL of GelStar Stain were added to it. After the stain was added, the mixture was poured into a gel mold inside an electrophoresis chamber and a gel comb was inserted. The mixture was allowed to harden into a gel inside the mold and then the comb was removed. TAE was then poured over the top of the gel until it reached the fill line inside the gel electrophoresis chamber. The mixtures used in the gel electrophoresis contained 2 µL of dye and varied amounts of sample and DEPC, depending on the concentration of an individual sample, to equal 20 µL total. Each well was loaded with 5 µL of one of the eight sample mixtures. Once all the wells were loaded, the gel electrophoresis apparatus was run on 100 V for 30 minutes. After the cycle was complete, the gel was removed from the gel electrophoresis apparatus and was photographed using a UV camera in a dark room. The picture taken of the gel was examined to determine if the integrity and quality of the total RNA were sufficient.

cDNA Synthesis

Using the isolated total RNA samples, preparations were made for cDNA synthesis. Before cDNA synthesis could be carried out, the total RNA had to first be treated with DNase. The DNase was diluted to 1/5 its initial concentration by combining 2 µL DNase, 1 µL of 10X PCR Buffer, and 7 µL DEPC. A DNase Master Mix was created for use with the eight mice liver samples. This mix consisted of 33.6 µL of 25 mM $MgCl_2$ and 3.2 µL of the 1/5 DNase. The DNase reactions were set-up in 8-strip tubes filled with 2 µg total RNA, 3.68 µL DNase Master Mix, and H_2O to equal a total volume of 20 µL. The reactions were carried out in a thermocycler by selecting subdirectory 1, program 1. This selection caused the thermocycler to run at 37° C for 30 minutes, 75° C for 10 minutes to inactivate the enzyme with heat, and a 4° C soak cycle at the end. The samples were then removed from the thermocycler and placed on ice. Next, cDNA synthesis (reverse-transcription) was performed. A 2 mg portion of lyophilized powder in a tube of pdN6 (random hexamers) was spun in a microfuge before adding 1.25 mL of DEPC to the tube and mixing it with the powder to create a solution. The solution was diluted once again by adding an equal-part DEPC in order to create a 0.8 mg/mL working solution. A RT Master Mix was then created using 200 µL 5X buffer, 100 µL DTT, 200 µL 10 mM dNTP mix, 100 µL pdN6, 190 µL DEPC, and 10 µL RTase. 80 µL of this mix were added to the 20 µL DNase-treated samples of RNA. The 100 µL samples were then put into the thermocycler and subdirectory 1, program 2 was selected. This program ran on 25° C for 10 minutes, 42° C for 50 minutes, and 72° C for 10 minutes. It was concluded by a 4° C soak cycle. The samples were then stored at -20° C.

Quantitative Real-time PCR

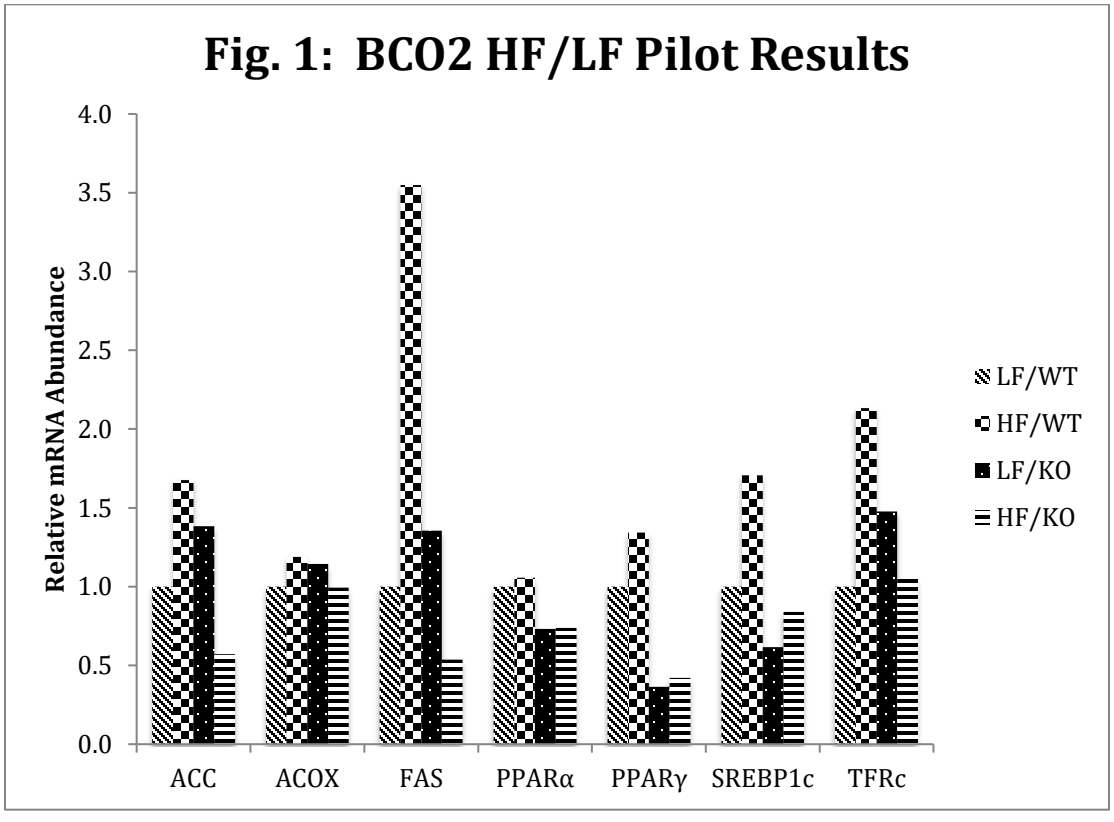
Quantitative real-time PCR (qPCR) is used for measuring variation in the level of gene expression between different samples. It is an extremely sensitive method and is able to determine these expression levels, even when there is low concentration of RNA. In this experiment, 5 μL of each isolated cDNA sample were used to perform qPCR. First, 2.5 μM working stocks that were prepared using 975 μL DEPC, 12.5 μL forward primer, and 12.5 μL reverse primer were utilized. A PCR Master Mix was then prepared for each of the eight genes being studied. These were 10 μL reactions that consisted of 10.2 μL DEPC, 4.8 μL Primer Mix, 20.0 μL SYBR Green, and 5.0 μL cDNA. A PCR plate was planned out on paper before being loaded with the samples to be run in triplicate. The PCR plate was then covered with a sealing film. These reactions were carried out in a thermal cycler that had been programed with sample names as well as with the desired genes to be detected.

For qPCR in this experiment, the samples obtained were run with the following proteins: cyclophilin B (Cyclo), peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor gamma (PPARγ), acyl coenzyme A oxidase (ACOX), acetyl coenzyme A carboxylase (ACC), sterol regulatory element-binding protein 1 (SREBP1c), fatty acid synthase (FAS), and transferrin receptor protein 1 (TFRc).

Results

For this experiment, the LF/WT group of mice is considered to be the control group. The graph below (Fig. 1) shows the relative mRNA abundance of each substance in relation to the LF/WT results, which were set at an mRNA abundance of 1.0. Expression of mRNA is

indicative of potential for protein to be synthesized. Increased relative mRNA abundance denotes increased probability that a particular protein will be formed. The graph below shows data for relative mRNA abundance of the genes being studied. Data for Cyclo is not shown on the graph, because Cyclo was used as the reference gene for this experiment. A reference gene is "an internal reaction control" that has a different sequence than the gene of interest [12]. The expression of reference genes should not be altered by experimental factors and should change very little between the organism's "tissues and physiological states" [12]. Reference genes ideally have threshold cycles like those of the target gene and show variability due to errors of technology or methods of preparation [12]. All data collected from this experiment was adjusted using the data for Cyclo as a standardization value.



ACC: The mRNA abundance for ACC in the LF/KO mice was 1.38, which is higher than in the LF/WT mice. The abundance of mRNA in the HF/KO mice was 0.57, which is significantly lower than the value of 1.68 for the HF/WT mice.

ACOX: The mRNA abundance for ACox1 was slightly higher in LF/KO mice at a value of 1.14 than it was in LF/WT mice. At 0.99, the mRNA abundance for HF/KO mice was somewhat lower than it was at 1.19 for HF/WT mice. Although the groups did not exhibit the same exact mRNA abundance, there were only slight variations between these four groups.

FAS: In relation to FAS, mRNA abundance was higher in the LF/KO mice at 1.35 than it was in the LF/WT mice control group. The HF/KO mice showed a significantly smaller abundance of mRNA than the HF/WT mice at 3.55 and 0.54, respectively. The contrast between these two groups with FAS showed by far the greatest discrepancy of any result in our study.

PPARs: The mRNA abundance for both PPARα and PPARγ was higher in the wild-type mice than in the BCO2 knockout mice, despite whether the mice had been fed a low-fat diet or a high-fat diet. There was a much larger variation between the knockout and wild-type mice relative to PPARγ than relative to PPARα. There was also very little difference between the LF/KO mice and the HF/KO mice in either one of the peroxisome proliferator-activated receptors. The HF/WT mice had a relative mRNA abundance of 1.06, only slightly differing from the LF/WT mice group. The HF/KO mice group had a relative mRNA

abundance of 0.74, barely differing from the LF/KO mice group value of 0.73. For PPARγ, the LF/KO mRNA abundance value was 0.36 and the HF/KO was somewhat similar at 0.42. The HF/WT value was much larger than any of the other values at 1.34.

SREBP1c: In connection with SREBP1c, the mRNA abundance was similar to the PPAR values in that abundance was higher in both wild-type mice than knockout mice, regardless of the type of diet they had been fed. In HF/WT mice, mRNA abundance was notably higher at 1.71 than in the other groups of mice. The LF/KO and HF/KO mice had mRNA abundance values of 0.62 and 0.84, in that order.

TFRc: With respect to TFRc, mRNA abundance was larger in LF/KO mice at 1.48 than in LF/WT mice. The mRNA abundance of HF/KO mice was much lower at 1.05 than the value of 2.13 in HF/WT mice.

Discussion

In the majority of the reactions studied, the HF/KO mice had a lesser mRNA abundance than the HF/WT mice, while the LF/KO mice has a greater mRNA abundance than their LF/WT counterparts. This suggests that in many cases, when the BCO2 gene is removed, mice that had been fed a high-fat diet were unable to make as much protein. In contrast, the majority of mice that were fed low-fat diets did not experience a lessening of mRNA abundance, potentially suggesting that BCO2 serves as a protective measure against the side effects of high levels of fat consumption. This could be through measures that were discussed earlier such as anti-inflammatory or anti-oxidative properties. To further

examine this theory, the qPCR results of each gene studied can be investigated in greater detail.

Correlations can be made between the results of several genes in this study. An example of this is demonstrated with ACC, FAS, SREBP1c. ACC is the rate-limiting enzyme in the first step in the biosynthesis of fatty acids [18]. FAS works after ACC has facilitated the carboxylation of acetyl-CoA to malonyl-CoA. It adds the malonyl-CoA molecules that are formed onto a primer in order to create a long chain fatty acid [14]. SREBP1c controls hepatic synthesis of fatty acids. It is an important regulator for the transcription of lipogenic enzymes like ACC and FAS [15]. In the HF/KO mice, there was decreased mRNA expression of ACC, FAS, and SREBP1c compared with the LF/WT mice. In the LF/KO and HF/WT mice, there is increased mRNA expression of ACC and FAS. In regards to SREBP1c, however, the HF/WT mice showed increased mRNA expression while the LF/KO mice showed decreased mRNA expression, suggesting that SREBP1c may be mostly impacted by the BCO2 knockout, rather than type of diet consumed. The decreased expression of ACC and FAS in the mice that were KO and/or HF suggests that a lack of BCO2 can impair fatty acid synthesis. If fatty acid synthesis is impaired, there are several problems that could occur. Fatty acids may not be able to be utilized as energy sources in the body or they may not be able to perform their structural and/or metabolic roles in the body. Diseases could also appear if an inability to oxidize fatty acids normally occurs due to lack of BCO2.

The PPARs also are related to fatty acid metabolism and were adversely impacted by the removal of BCO2 in the study. PPARα is crucial to the regulation of genes that are relative to hepatic "peroxisomal, mitochondrial and microsomal fatty acid oxidation" and works to maintain normal levels of plasma triglycerides [15]. Because both the LF/KO and

HF/KO mice showed decreased mRNA expression of PPARα, it can be hypothesized that absence of the BCO2 enzyme could lead to an inability to regulate plasma triglycerides. High triglycerides can lead to poor-health conditions such as atherosclerosis that are associated with inflammation and oxidative stress. Because the level of PPARα expression is higher in the presence of BCO2, the previously discussed role of BCO2 as a reducer of oxidative stress is supported. There was virtually no difference between the LF/KO and HF/KO groups or between the LF/WT and HF/WT groups, suggesting that the difference between groups is based exclusively on the absence or presence of BCO2 in regards to PPAR α. The level of fat consumed by mice in relation to PPARα did not appear to affect the ability of proteins to be made. PPARγ plays a large role in regulating lipid metabolism and adipocyte differentiation. It is also an important "modulator of inflammatory and immune responses" [16]. The expression of PPARγ is decreased in both LF/KO and HF/KO mice. Because of the role PPARγ plays in guarding against inflammation, its reduction in the absence of BCO2 further implies that BCO2 likely acts as an anti-inflammatory agent. Like PPARα, there was not much difference between the knockout mice that ate high-fat and low-fat diets. However, there was a more of a difference between the wild-type mice that consumed low-fat and high-fat diets in PPARγ than in PPARα, suggesting that level of fat consumption may alter the ability of PPARγ to be made.

While connections could be made between many of the genes in this experiment, in other cases, the results of a particular gene did not suggest an identifiable positive correlation with other results from the study or with BCO2. Two examples of this can be seen with ACOX and TFRc. ACOX catalyzes the first step in the beta-oxidation of long-chain and very-long-chain fatty acyl-coenzymes in the peroxisome [17]. The mRNA abundance

results for ACOX had no clear pattern in regards to type of diet consumed or the presence or absence of the BCO2 gene. The results for all four groups of mice in regards to ACOX were similar. Because the results of our study did show evidence of a clear relationship between BCO2 and ACOX, there is not reason to believe that BCO2 has a direct effect on the fatty acid beta-oxidation pathway, at least in regards to the first step. Our results also failed to identify connections with TFRc and BCO2. TFRc is a cell surface receptor that functions in iron homeostasis by controlling the amount of iron that is taken up by transferrin, the serum protein involved with synthesis of hemoglobin, to be delivered to cells [18]. TFRc was studied in order to determine whether or not the BCO2 enzyme seemed to have an effect on iron metabolism. The data we collected did not establish a pattern that would suggest a correlation between the presence of BCO2 and iron homeostasis. This result was surprising because of the connection with impaired iron metabolism and vitamin A deficiency. I had anticipated that TFRc would be adversely impacted in the knockout mice because the removal of BCO2 would theoretically lead to a lack of vitamin A synthesis due to a shortage of carotenoids being cleaved. Although a clear connection could not be made between BCO2 and iron metabolism, the expression of TFRc was much higher in the HF/WT mice than any of the other groups, possibly suggesting a link between a high level of fat consumption and iron uptake.

Conclusions

The results of this study indicated that BCO2 indeed plays other roles in the body besides vitamin A synthesis. Our results showed that BCO2 knockout adversely impacted expression of enzymes involved in fatty acid synthesis. This suggests that BCO2 is an

important regulator of fatty acid synthesis and its absence could have a negative impact on health. In addition to enzymes involved in fatty acid synthesis, enzymes involved in inflammatory and immune responses were also adversely impacted by the absence of BCO2. This suggests that in addition to impaired fatty acid regulation, other inflammatory conditions could ensue due to BCO2 knockout. In some cases, the effects of BCO2 knockout were amplified by consumption of a high-fat diet, while in other cases, BCO2 knockout had a substantial impact regardless of the type of diet consumed by the mice. This suggests that BCO2 knockout could be responsible for metabolism issues and health concerns in individuals, even if they are being responsible in regards to the amount of fat in their diets.

Questions for Future Study

In the future, I believe it would be beneficial to study the effects of a greater variety of fat consumption percentages with mice in the presence and absence of BCO2. This would be particularly interesting in enzymes such as FAS that were impacted differently by BCO2, depending on whether they consumed a HF or LF diet. Would examining different percentage levels reveal a turning point where BCO2 begins to impact the expression of these proteins differently?

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